GONAD DEVELOPMENTAL CYCLE
3.0. Introduction

The success of any fish species is ultimately based upon the ability of its members to reproduce successfully in a fluctuating environment. One of the defining features of a species, therefore, is its reproductive strategy, as reflected in anatomical, behavioral, physiological and energetic adaptations (Moyle and Cech, 2000).

Maturation refers to cyclic morphological changes, which the male and female gonads undergo to attain full growth and ripeness. The development of the piscine gonads has been described in terms of stages of maturity (Treasurer, 1990; Ha and Kinzie, 1996). The spawning season of a species can be determined by the macroscopic and microscopic examination of the gonads (Pen et al., 1993; Ha and Kinzie, 1996).

Fish ontogeny is usually divided into different stages. They are larval, pre-reproductive, and reproductive and post-reproductive periods (Rass, 1989; Kryzhanovsky, 1949; Balon, 1984). The pre-reproductive period is characterized by the formation and maturation of oocytes. This is followed by ovulation and fertilization. Fish ontogeny is influenced largely by environmental conditions (Alm, 1959; Stearns and Crandall, 1984).

Asynchronous development of the ovary (Wallace and Selman, 1981) is common in cyprinodonts like Fundulus heteroclitus (Able and Hagan, 2003), Fundulus lucinae (Kneib, 1978), Epilampus bifasciatus (Guma’a, 1982) and Lucania goodie (Fuller, 2002).

Oogenesis was described in several ornamental fishes like Poecilia reticulate (Monaco et al., 1978), Gambusia holbrooki (Thibaut et al., 2002) and Gambusia affinis (Koya et al., 2003; 2004). Spermatogenesis was studied in ornamental fishes like Poecilia reticulate (Monaco et al., 1978), Macrodus cupanus (Jacob and Nair, 1983),
Barbus sps. (De Silva et al., 1985), Puntius dukai (Joshi and Joshi, 1989), Esox lucius (Treasurer, 1990) and Gambusia affinis (Koya et al., 2003; 2004).

In killifishes the development of oocyte had been followed in Fundulus grandis (Greeley et al., 1988), Aphyosemion splendopleure (Thiaw and Mattei, 1991), Aphanius fasciatus (Leonardos and Sinis, 1998) and Fundulus heteroclitus (Sharpe et al., 2004). Spermatogenesis was studied in Aphanius dispar (Hawawi and Imam, 1984), Aphyosemion sps. (Thiom et al., 1996) Aphanius fasciatus (Leonardos and Sinis, 1998) and Fundulus heteroclitus (Pait and Nelson, 2003; Fabra and Cerda, 2004).

A thorough analysis of the literature showed that no detailed study had been carried out in the killifish A. lineatus. Hence the present study was undertaken to provide in-depth information on the gonadal cycle of A. lineatus. Oocyte-size frequency distribution was also analyzed to pin-point the spawning season.

3. 1. Materials and methods
3. 1. 1. Maturity analysis

The males and females of killifish A. lineatus were randomly selected and standard length (SL) and total length (TL) were measured. Total length was measured from the tip of the lower jaw to the tip of the longest ray of the caudal fin. The length measurements were taken to the nearest millimeter and weight to the nearest milligram. Gonads were usually removed from the fish within a few hours of capture, and their sex and stage of reproductive maturity were determined using a macroscopic staging system. The criterion for the identification of the maturity stage was based on colour, texture, size, shape and extent of occupancy of gonads in the body cavity (Mc Bride et al., 2002). The GSI was calculated by using the following formula (Mc Bride et al., 2002):
GSI = GW/ (BW-GW) x 100

where GW is the weight of the gonad (g) and BW is the wet weight of the fish (g).

3.1.2. Histological technique

A portion of tissue was excised from the middle of either the right or left gonad fixed in Bouin’s fluid and dehydrated in a series of increasing alcohol concentrations. The tissue was embedded in paraffin wax sectioned along the transverse plane (6 - 8 μm), stained with Haematoxylin eosin or Masson’s or Mallory’s triple stain (Quintero-Hunter et al., 1991). The histological classification of maturity stages was based on changes in the diameter of unspawned ova, degree of yolk deposition and proportion of oocytes ready for ovulation.

3.1.3. Measurement of oocyte diameter

Oocyte diameters were measured in the randomly selected female gonads. A sub-sample of the ovarian tissue was removed and the oocytes were teased apart with needles in a petri dish filled with glycerol diluted to 30% with water. The diameters of 100 - 250 oocytes per fish were measured by means of an ocular meter fitted in a binocular microscope (Mc Bride et al., 2002).

3.1.4. Frequency distribution of oocytes

The number of oocytes in each size class was counted and expressed as a percentage of the total number of oocytes. The average oocyte diameter was determined for each growth stage. The egg size was measured as the maximum diameter along two axes. A total of 100 oocytes measurements were made from each ovary and around 25 ovaries were measured each fortnight and averaged for monthly diameter values.

The developmental stage of oocytes was observed in each female in increasing order, as chromatin nucleolar, perinucleolar, cortical alveolar, vitellogenic, nucleus...
migration and nucleus breakdown stages (West, 1990). The regressed ovaries distinguished from maturing ovaries by the presence of melano macrophage centers. The degree of gamete development in crypts, the level of spermatozoa storage in the central duct were used as criteria in staging male maturity (Mc Bride et al., 2002).

3. 2. Results

3. 2. 1. Ovarian development

The ovary of *A. lineatus* was a paired saccular body with the investing wall of peritoneum, which led to the formation of ovisacs. Each ovary remained suspended in the body cavity by a mesentery, called mesovarium. The anterior portion of the ovisac ended blindly, but the posterior portion continued into an oviduct. The ovary having such continuity of its lumen to the oviduct was known as cystovarian ovary.

During the breeding season, the ovisacs became enlarged in size due to accumulation of a large number of ova. The ripe ova were discharged into the central ovarian cavity and from there they passed down the oviduct directly to the exterior through the genital pore. The oviduct had separate opening from the urinary and anal openings. In immature and juvenile fishes the ovaries were symmetrical. But once they became matured one lobe was shorter than the other. The gonadosomatic index of *A. lineatus* increased (4.365%) with the progressive development of the gonads in females, until they became ripe and ready to spawn. Once they spawned the eggs, the GSI index declined (2.054%) in spent or regressed conditions of fish. An increased ovarian weight was observed in immature (0.015g), mature (0.024g) and ripe ovaries (0.039g) (Figure 19). The seasonal variation in the gonadosomatic index was well marked in *A. lineatus*. The morphological appearance of the ovaries of *A. lineatus* allowed the categorization of five maturity stages (Figure 20 and Plate 2). The characteristics of each maturity stage
Figure 19: *Apocheilus lineatus*: Relationship between the ovary weight and the gonadosomatic index with stages of maturity (Values ± S.E)
Figure 20  *Aplocheilus lineatus*: Gonadal cycle

- Immature
- Developing/Maturing stage
- Partially Spent
- Hydrated/Ripe Stage
- Mature
- Spent
Plate - 2. Oogenesis of *Aplocheilus lineatus*

a. Immature ovary showing numerous unyolked oocytes (u) and few partially yolked (p) oocytes. X150, Mallory’s Triple stain.

b. Immature ovary showing numerous unyolked oocytes (u) and few partially yolked (p) oocytes. X150, Mallory’s Triple stain.

c. Maturing ovary showing numerous yolked oocytes (y), germinal zone (GZ) and few residual atretic oocytes (AO). X150, Mallory’s Triple stain.

d. Maturing ovary showing numerous yolked oocytes (y) and few residual atretic oocytes (AO). X150, Mallory’s Triple stain.

e. Mature ovary showing numerous yolked (y) and hydrated (H) oocytes and few unyolked (u) and partially yolked (p) oocytes and (O) oogonial nests. X150, Mallory’s Triple stain.

f. Mature ovary showing numerous yolked (y), hydrated (H) oocytes and few unyolked (u), partially yolked (p) oocytes, and (O) oogonial nests X150, Mallory’s Triple stain.

g. Spent ovary showing many post ovolatory follicles (POF) and fewer unovulatory follicles (UOF) X150, Mallory’s Triple stain.

h. Spent ovary showing many post ovolatory follicles (POF) and fewer unovulatory follicles (UOF) X150, Mallory’s Triple stain.
are described in Table 21. The distinctive features of ovarian developmental stages are as follows:

a. **Immature**: The immature ovary was small, slender, ribbon like, conical and tapering behind. It was dull white, transparent and occupied one-third of the abdominal cavity. The oocytes were invisible to the naked eye. Both the ovarian lobes were of the same length.

b. **Maturing**: The maturing ovary was translucent and whitish yellow in colour. It was elongated and oval in transverse section. The ovary occupied half of the abdominal cavity. Eggs were visible under the magnifying lens. One lobe was shorter than the other.

c. **Mature**: The mature ovary was yellowish white with visible blood capillaries which occupied two-thirds of the abdominal cavity. Eggs were large and visible to the naked eye. Ova were not free in the lumen. One left lobe was half the length of the right lobe.

d. **Partially Spent**: The partially spent ovary was whitish red and the spent region appeared translucent which occupied half of the abdominal cavity. Ova were free in the lumen and they came out when a little pressure was applied on the abdomen. Few ova were seen in the spent region.

e. **Regressed**: Ovaries were dirty white and translucent and look flaccid and shrunken and contained a few translucent eggs. Many ova were found in a reabsorbing / degenerating condition.

The frequency distribution of various ovarian stages in different length groups of *A. lineatus* during various months is depicted in Figure 21. During the pre-breeding season maturing and mature fishes of 45 - 48mm TL were 40 - 48% during the breeding season mature and gravid fishes with 50 - 60mm TL were 55 - 65% and at post-breeding season the regressed females were maximum (55 - 68%).
<table>
<thead>
<tr>
<th>MATURITY STAGES</th>
<th>FISH</th>
<th>OVARY</th>
<th>GSI RANGE (%)</th>
<th>GROSS ANATOMICAL FEATURES</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TL (mm)</td>
<td>WEIGHT (gm)</td>
<td>LENGTH (cm) (R / L)</td>
<td>WEIGHT (gm)</td>
</tr>
<tr>
<td>Immature</td>
<td>25 to 35</td>
<td>0.23-0.45</td>
<td>0.2 / 0.2</td>
<td>0.003-0.007</td>
</tr>
<tr>
<td>Maturing</td>
<td>36-45</td>
<td>0.42-0.98</td>
<td>0.4 / 0.3</td>
<td>0.010-0.025</td>
</tr>
<tr>
<td>Mature</td>
<td>46-70</td>
<td>0.853-2.35</td>
<td>0.6 / 0.3</td>
<td>0.022-0.085</td>
</tr>
<tr>
<td>Regressed</td>
<td>53-65</td>
<td>2.10-2.25</td>
<td>0.4 / 0.3</td>
<td>0.007-0.015</td>
</tr>
</tbody>
</table>
Figure - 21: *Aplocheilus lineatus*: Seasonal occurrence of ovarian maturity stages among different length groups of female
3.2.2. **Oocyte development**

The developmental stage of oocytes was determined by a microscopic examination of the ovary. The oogenesis of *A. lineatus* was categorized into eleven developmental stages from oogonia to atretic oocyte (four sub stages). The nomenclature and classification of oocyte stages were based on Wallace and Selman (1981) and West (1990). The classification of oocytes based on cytomorphological features is given in Table 22. A detailed description of the different stages of oocytes is presented in Table 23.

1. **Oogonia:** Oogonia were seen in maturing and regressed ovaries. They were found singly or in small nests. Oogonia had a diameter of 80 - 95 μm with a nucleus which stained deep orange with Mallory's trichrome (Plate 3a; Plate 4a). They had very little cytoplasm with a prominent nucleolus.

2. **Chromatin nucleolar oocyte (CNO):** The CNO had a large nucleus surrounded by a thin layer of cytoplasm with a diameter of 98 - 125 μm. Prefollicle cells surrounded each oocyte (Plate 3b; Plate 4b, c, d, e). The nucleus consisted of single, large nucleoli.

3. **Perinucleolar oocyte (PNO):** Concomitant with oocyte growth, the nucleus increased in size. Many nucleoli appeared at the periphery (Plate 3c, d; Plate 4f, g). The cytoplasm stained uniformly (diameter 137-149 μm). The chromatin nucleolar and perinucleolar stages were referred to as primary growth phase (Wallace and Selman, 1981) or first growth phase (Forberg, 1982).

4. **Cortical alveolar oocyte (CAO):** This stage was characterized by the appearance of vacuoles in the cytoplasm (diameter 145-222 μm). This also was termed as primary yolk stage (Plate 3f; Plate 4h). With conventional H & E preparations, these vacuoles appeared empty (Forberg, 1982). The cytoplasmic vacuoles aggregated to form cortical alveoli. The yolk granules appeared in the perinuclear region.
Table 22. *Aplucheilus lineatus*: Comparative features of macroscopic and microscopic stages of the ovary

<table>
<thead>
<tr>
<th>OVARIAN STAGE</th>
<th>MACROSCOPIC STAGES</th>
<th>MICROSCOPIC STAGES</th>
<th>OOCYTE DIAMETER RANGE (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Unyolked oocyte</td>
<td>Immature and previtellogenic</td>
<td>80 - 150</td>
</tr>
<tr>
<td>II</td>
<td>Partially yolked oocyte</td>
<td>Early vitellogenic Oocytes</td>
<td>138 - 280</td>
</tr>
<tr>
<td>III</td>
<td>Yolked oocyte</td>
<td>Vitellogenic and mature Ova</td>
<td>275 - 618</td>
</tr>
<tr>
<td>IV</td>
<td>Hydrated Oocyte</td>
<td>Preovulatory and unovulatory follicles</td>
<td>602 - 915</td>
</tr>
</tbody>
</table>
Table 23. *Aplacheilus lineatus*: Cytomorphological features of oocyte development.

<table>
<thead>
<tr>
<th>OOCYTE STAGE</th>
<th>OOCYTE DEVELOPMENTAL STAGE</th>
<th>NUCLEAR FEATURES</th>
<th>CYTOPLASMIC FEATURES</th>
<th>NUCLEOLUS</th>
<th>OOCYTE DIAMETER RANGE (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Oogonia</td>
<td>Single</td>
<td>Thin cytoplasmic layer</td>
<td>Single</td>
<td>80-95</td>
</tr>
<tr>
<td>II</td>
<td>Chromatin nucleolar oocyte</td>
<td>Single large nucleus</td>
<td>Thin cytoplasmic layer</td>
<td>Single dark stained</td>
<td>98-125</td>
</tr>
<tr>
<td>III</td>
<td>Perinucleolar oocyte</td>
<td>Large germinal vesicle</td>
<td>Cytoplasm increased and became bulky</td>
<td>Few, peripheral</td>
<td>137-149</td>
</tr>
<tr>
<td>IV</td>
<td>Cortical alveolar oocyte</td>
<td>Central germinal vesicle</td>
<td>Appearance of cortical Alveoli</td>
<td>Many</td>
<td>145-222</td>
</tr>
<tr>
<td>V</td>
<td>Early vitellogenic oocyte</td>
<td>Central germinal vesicle</td>
<td>Appearance of yolk granules</td>
<td>Many</td>
<td>202-235</td>
</tr>
<tr>
<td>VI</td>
<td>Vitellogenic oocyte</td>
<td>Central germinal vesicle</td>
<td>Appearance of yolk vesicles in the peripheral cytoplasm</td>
<td>Many</td>
<td>217-243</td>
</tr>
<tr>
<td>VII</td>
<td>Late vitellogenic oocyte</td>
<td>Central germinal vesicle</td>
<td>Appearance of yolk platelets</td>
<td>Many</td>
<td>235-279</td>
</tr>
<tr>
<td>VIII</td>
<td>Germinal vesicle migration (GVM) oocyte</td>
<td>Peripheral germinal vesicle, dissolution of the membrane</td>
<td>Coalescence of yolk platelets</td>
<td>Many</td>
<td>386-568</td>
</tr>
<tr>
<td>IX</td>
<td>Germinal vesicle breakdown (GVBD) oocyte</td>
<td>Germinal vesicle at animal pole</td>
<td>-</td>
<td>Many</td>
<td>465-623</td>
</tr>
<tr>
<td>X</td>
<td>Hydrated oocyte</td>
<td>-</td>
<td>Compaction of yolk platelets</td>
<td>Invisible</td>
<td>615-704</td>
</tr>
<tr>
<td>XI</td>
<td>Follicular atresia</td>
<td>-</td>
<td>Hypertrophied of granulosa cells</td>
<td>-</td>
<td>83-677</td>
</tr>
</tbody>
</table>

(all stages)
Plate - 3. Developmental morphology of oocytes of *Aplocheilus lineatus*

a. Oogonia - (cm) chromatin, (nu) nucleolus. X 400 Masson’s Triple stain.

c. Perinucleolar oocyte (PNO), (cm) chromatin, (cy) cytoplasm, (yn) yolk nucleus and (nm) nuclear membrane X 1000 Flemming’s Triple stain.

e. Primary oocyte (PO) (cm) - chromatin, (cy) - cytoplasm, (yn) - yolk nucleus, hyaline membrane (hm) X 400 HE.

b. Chromatin nucleolar oocyte (nu) multiple nucleoli, X 400 Masson’s Triple stain.

d. Perinucleolar oocyte (PNO), (cm) chromatin, (cy) cytoplasm, (yn) yolk nucleus, (hm) hyaline membrane. X 1000 Flemming’s Triple stain.

f. Cortical alveolar oocyte (CAO), (ca) cortical alveoli, (cy) cytoplasm, (gv) germinal vesicle. X 1000 Masson’s Triple stain.

h. Vitellogenic oocyte (VO), yolk granules (yg), germinal vesicle(gv). X 400 Mallory’s Triple stain.

g. Vitellogenic oocyte (VO), yolk granules (yg), germinal vesicle(gv), X 400 Masson’s Triple stain.
Plate - 4. Developmental morphology of oocytes of *Aplocheilus lineatus*

a. Sectional view of the ovary showing 'oogonial nest (O), X 400 Flemming's Triple stain.

b. Chromatin nucleolar oocyte (CNO) showing single nucleolus (nu) and hyaline membrane (hm), chromatin (cm) X 400 Masson's Triple stain.

c. Chromatin nucleolar oocyte (CNO) showing four nucleoli (nu), X 400 Masson's Triple stain.

d. Chromatin nucleolar oocyte (CNO) showing eight nucleoli (nu), X 400 Mallory's Triple stain.

e. Chromatin nucleolar oocyte (CNO) showing eight nucleoli (nu), X 400 Mallory's Triple stain.

f. Perinucleolar oocyte (PNO) showing multiple nucleoli (nu), chromatin (cm), cytoplasm (cy) X 400 Mallory's Triple stain.

g. Perinucleolar oocyte (PNO) showing multiple nucleoli (nu), chromatin (cm), cytoplasm (cy) X 400, Mallory's Triple stain.

h. Cortical alveolar oocyte (CAO) showing cortical granules (cg), ooplasm (op) and cortical alveoli (ca). X 400, Masson's Triple stain.
Plate - 5: Magnified view of single vitellogenic oocyte, germinal vesicle (gv), yolk granules (yg), granulosa layer (gl) and vitelline membrane (vm), X 400 Masson’s Triple Stain.
5. Early vitellogenic oocyte (EVO): In this stage the yolk globules began to appear between the yolk granules in the peripheral cytoplasm, which marked the initiation of vitellogenesis (diameter 202 - 235μm) (Plate 3g; Plate 6a, b, c). The yolk globules were minute, spherical and acidophilic.

6. Late vitellogenic oocyte (LVO): The cytoplasm was packed with acidophilic yolk globules (diameter 235 - 279μm) (Plate 3b; Plate 6d, e). As the yolk globules increased in size the oocytes became large. Lipid bodies situated in the perinuclear cytoplasm fused with one another and became large.

7. Germinal vesicle migration stage (GVM): The GVM stage was indicated by the peripheral migration of the nucleus and the dissolution of nuclear membrane (diameter 386 - 568 μm). The nucleus progressively displaced towards the animal pole where the micropyle was located (Plate 6f, g, h). The release of the first polar body followed before the oocyte was ovulated into the lumen. The follicular layer became stretched, forming wide intercellular spaces.

8. Germinal vesicle breakdown stage (GVBD): After the migration of the nucleus to the periphery, its nuclear envelope broke down (germinal vesicle break down GVBD) (diameter 465 - 623μm) (Plate 7a, b, c). The process of coalescence further resulted in the formation of a continuous mass of yolk in the central part of the oocyte. Yolk globules of various sizes remained in the peripheral cytoplasm, but appeared to be reduced in number as the yolk mass increased.

9. Hydrated oocyte (HO): This was identified by the rapid uptake of fluid by the follicle (Fulton, 1898). This started when the nucleus had completed its migration to the animal pole (Plate 7d). The yolk globules began to fuse, forming yolk plates. The nucleus was not clearly visible. The nucleus havin reached the animal pole, the nuclear membrane disintegrated, dispersing its contents into the cytoplasm. All yolk globules fused into plates and the oocyte expanded greatly, stretching the granulosa and thecal layers (diameter 615 - 704μm).
Plate - 6. Developmental morphology of oocytes of *Aplocheilus lineatus*

a. Vitellogenic oocyte (VO) yolk granules (yg), germinal vesicle (gv), granulosa layer (gl), vitelline membrane (vm) X 400 Mallory's Triple Stain.

b. Vitellogenic oocyte (VO), (gv) germinal vesicle, (yg) yolk granules, (vm) vitelline membrane, (gl) granulosa layer, (tl) thecal cell layer X 400 Flemming's Triple Stain.

c. Vitellogenic oocyte (VO), granulosa cell layer (gl), thecal cell layer (tl), chorion (ch) and (vm) vitelline membrane X 1000 Mallory's Triple Stain.

d. Vitellogenic oocyte (VO) showing (yg) yolk granules, granulosa cell layer (gl), thecal cell layer (tl) and chorion (ch) X 1000 Mallory's Triple Stain.

e. Vitellogenic oocyte showing vitelline membrane (vm), granulosa cell layer (gl), thecal cell layer (tl) and chorion (ch) X 1000 Masson's Triple Stain.

f. Final oocyte maturation (FOM), (HO) Hydrated oocyte, (vm) vitelline membrane, (gl) granulosa cell layer X 400 Mallory's Triple Stain.

g. Final oocyte maturation (FOM), (HO) Hydrated oocyte, X 400 Mallory's Triple Stain.
Plate - 7. Developmental morphology of oocyte of *Aplocheilus lineatus*

a. Germinal vesicle breakdown (GVBD), (vm) vitelline membrane, (gl) granulosa layer, (tl) thecal layer and (yp) yolk platelets X 200 Masson’s Triple stain.

c. Germinal vesicle breakdown (GVBD) X 400 Masson’s Triple stain.

e. α atresia of unyolked oocytes consisting of chromatin nuclear oocyte (CNO), Perinuclear oocyte (PNO), granulosa (interior) cells (gic) X 100 Masson’s Triple stain.

b. Germinal vesicle breakdown (GVBD), (vm) vitelline membrane, (gl) granulosa layer, (tl) thecal layer and (yp) yolk platelets X 200 Masson’s Triple stain.

d. Hydrated oocyte (HO), (vm) vitelline membrane, (gl) granulosa layer, (tl) thecal layer X 1000 Masson’s Triple stain.

f. α atresia of unyolked oocytes consisting of chromatin nuclear oocyte (CNO), Perinuclear oocyte (PNO), granulosa (interior) cells (gic) X 100 Masson’s Triple stain.
3.2.3. Characteristics of oocytes undergoing maturation in vivo

The appearance of oocytes within their follicles undergoing maturation and ovulation is indicated in Plate 13d. As the oocyte enlarged, the clearly visible GV (Plate 13e) migrated to the periphery (Plate 13f) and disappeared (Plate 13i). At the same time, the oocyte became more translucent and peripherally attached oil droplets gradually coalesced with one another (Plate 14a). Eventually, the oil droplets lost their peripheral attachment and were able to move freely throughout the oocyte; at this stage they collected at the upper surface (Plate 14b). After this stage was reached, the oocyte ovulated out of its follicle into the ovarian lumen. GVBD in vivo generally occurred when the follicle reached a diameter of 465 to 623µm.

3.2.4. Follicular atresia

Oocyte degeneration or oocyte atresia of A. lineatus was categorized into four stages (Table 24). The frequency of occurrence varied seasonally (Figure 22). The nomenclature and general characteristics are defined by Lambert (1970a), but the details of the description of individual stages are based on Hunter and Macewicz (1985).

a) Alpha atresia of unyolked oocyte: The nucleus first disintegrated and the zona pellucida broke down (Plate 7e, f). The granulosa cells became hypertrophied and phagocytised the cytoplasmic materials.

b) Alpha stage atresia of yolked oocyte: In the alpha stage of atresia the oocyte was resorbed, leaving only the follicular layer (Plate 8b, c, d, e, f). The early phase of alpha stage atresia was characterized by the disintegration of the nucleus, fragmentation of the zona pellucida and the dissolution of some of the yolk globules.

c) Beta stage atresia: Initially the beta-stage atretic follicle was a compact structure composed of numerous disorganized granulosa cells surrounded by a thin thecal layer (Plate 9a, b). The nucleus of the granulosa cells was pycnotic and many of the cells contained intracellular vacuole(s).
Table 24. *Aplocheilus lineatus*: Various stages of follicular atresia.

<table>
<thead>
<tr>
<th>STAGE</th>
<th>ATRESIA TYPE</th>
<th>MICROSCOPIC FEATURES</th>
<th>DIAMETER RANGE (μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>α-atresia -unyolked oocyte</td>
<td>Disintegration of nucleus, dissolution of zona pellucida, hypertrophied granulosa cells.</td>
<td>85-200</td>
</tr>
<tr>
<td>II</td>
<td>α-atresia —yolked oocyte</td>
<td>Disintegration of yolk globules, hypertrophied granulosa cells.</td>
<td>215- 355</td>
</tr>
<tr>
<td>III</td>
<td>β-atresia — yolked oocyte</td>
<td>Phagocytic granulosa cells. Appearance of intracellular vacuoles</td>
<td>328- 525</td>
</tr>
<tr>
<td>IV</td>
<td>γ- atresia -yolked oocyte</td>
<td>The granulosa cells contained flocculent material</td>
<td>515- 603</td>
</tr>
<tr>
<td>V</td>
<td>δ- atresia –yolked oocytes</td>
<td>The presence of dark yellowish-brown, fine granular pigment in the granulosa cells.</td>
<td>535- 627</td>
</tr>
</tbody>
</table>
Figure 22: *Aplocheilus lineatus*: Frequency distribution of follicular atresia
Plate - 8: Oocyte undergoing atresia in the killifish Aplocheilus lineatus.

a. Breaking down of yolk granules. Yolk globule (yg), nucleus (n), and yolked alpha atresia (ya), X 400 Mallory’s Triple stain.

b. Yolked oocyte undergoing atresia. Note hypertrophied granulosa (g) and thecal cells (t) X 1000 Mallory’s Triple stain.

c. α atresia of yolked oocyte showing disintegration of nucleus(n), X 400 Masson’s Triple stain.

d. α atresia of yolked oocyte showing disintegration of yolk globules (yg), nucleolus (nu), granulosa layer (g) and thecal cells (t)X1000 Mallory’s Triple stain.

e. α atresia of yolked oocyte showing hypertrophied granulosa cell layer (g) X 400 Masson’s Triple stain.

f. α atresia of yolked oocyte showing invasion of melanomacrophages (mc) X 400 Mallory’s Triple stain.
d) **Gamma stage atresia:** The gamma-stage atretic follicle was usually much smaller than the beta stage follicle (Plate 9c, d). The granulosa cells contained flocculent material of light-yellow hue, and had nuclei of irregular shape. The granulosa cells were surrounded by fewer thecal cells.

e) **Delta stage atresia:** The diagnostic characteristic of this stage was the presence of a dark yellow-brown, finely granular pigment in the granulosa cells (Plate 9e, f). The delta-stage atretic follicles were normally very small structures typically composed of 2 - 20 granulosa cells in the connective tissue stroma. Thecal cells and blood vessels no longer directly encompassed the granulosa cells because they were absorbed into the ovarian connective tissue stroma.

### 3.2.5. Frequency distribution of oocytes

The ovary of *A. lineatus* contained almost all the stages of developing oocytes from 80 to 925μm in diameter. But the frequency of occurrence varied seasonally (Figure 23). Atretic oocytes were observed in all stages, *i.e.*, primary, vitellogenic and mature ova and the sizes ranged from 83 to 670μ.

### 3.2.6. Testicular cycle

Testes in *A. lineatus* were paired saccular bodies situated on either side, ventral to the kidneys along the posterior region of the abdominal cavity. A fold of peritoneum, enclosing a coelomic space, became continuous with the testis of each side, and was termed mesorchium. The sperm duct kept the continuation of its anterior part deep inside the testis and there it gave branching and sub-branching to form a system of tubules for collecting the sperm. At maturity the testes were long, tubular ribbon like structures with a maximum length of 10mm and weight of 0.012gm.

The gonadosomatic index increased progressively with the development of the testes in males until they became mature and ripe and declined sharply as they spawned (spent). The increase in weight of the testes in various maturity stages is depicted in
Plate - 9: Oocyte undergoing atresia in the killifish *Aplocheilus lineatus*.

a. β atresia of yolked oocyte showing disorganized granulosa cells (g). X 1000 Masson’s Triple stain.

b. β atresia of yolked oocyte showing formation of atretic capsule (ac). X 1000 Masson’s Triple stain.

c. γ atresia of yolked oocyte showing irregularly shaped atretic follicle (AF). X 400 Masson’s Triple stain.

d. γ atresia of yolked oocyte showing shrunken atretic follicle (AF). X 400 Masson’s Triple stain.

e. δ atresia of yolked oocyte showing complete invasion of granulosa cells (g). X 400 Masson’s Triple stain.

f. δ atresia of yolked oocyte showing (AF) atretic follicle enclosing granulated pigments (gp). X 100 HE.
Figure 23: *Aplocheilus lineatus*: Frequency distribution of oocyte developmental stages.
Figure 24. The testes of *A. lineatus* exhibited well marked seasonal changes and the testicular cycle was categorized into four stages. The histology of reproductive stages of *A. lineatus* is given in Table 25. The morphological characteristics of each maturity stage are given in Table 26.

1. **Immature**: The testes of immature *A. lineatus* were thin, slender thread like translucent structures of dull white colour.

2. **Ripening**: The testes were creamy white in colour and showed gradual increase in volume and became opaque. Milt did not come out even on the application of pressure on the abdomen. They extended to 1/3 rd of the abdominal cavity.

3. **Ripe**: The testes were milky white in colour and extended to a half of the abdominal cavity. Milt oozed on application of a slight pressure on the abdomen.

4. **Spent**: Testes were blood shot and flaccid. They appeared like inflated air sacs under the microscope.

The percentage frequency of various maturity stages of the testicular cycle as a function of total length of the male *A. lineatus* during the period of study is shown in Figure 25. During the pre-breeding period the immature and maturing males of 36 - 50mm TL were 55 - 60%, during the breeding season mature and ripe males of 45 - 65mm TL were 50 - 60% and during the post-breeding season the spent and partially spent males of 48 - 63mm TL with flaccid testes were 45 - 60%.

3. 2. 7. **Spermatogenesis**

The spermatogenesis in the testes of *A. lineatus* was categorized into five stages based on the histo-morphological characteristics. The histological features of the spermatogenic stages are presented in Table 27.
Figure: 24: *Aplocheilus lineatus*: Relationship between the testis weight and the gonadosomatic index with stages of maturity (Values ± S.E)
Table 25. *Aplocheilus lineatus*: Ovarian and testicular histology of reproductive stages

<table>
<thead>
<tr>
<th>MATURITY STAGE</th>
<th>OVARIAN HISTOLOGY</th>
<th>TESTICULAR HISTOLOGY</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immature</td>
<td>Numerous oogonia and few primary oocytes</td>
<td>Only Spermatogonia seen.</td>
</tr>
<tr>
<td>Maturing</td>
<td>Early vitellogenesis: many Primary oocytes, oogonia and oocytes with yolk vesicles</td>
<td>Early spermatogenesis, few scattered spermatocytes.</td>
</tr>
<tr>
<td>Mature</td>
<td>Late vitellogenesis: Oogonia, Many vitellogenic and hydrated oocytes.</td>
<td>Late spermatogenesis, Spermatozoa collecting in tubules and central lumen, Melano macrophages also present.</td>
</tr>
<tr>
<td>Regressed</td>
<td>Oogonia, primary oocytes, few vitellogenic oocytes, Post ovulatory follicles and atretic oocytes seen.</td>
<td>-</td>
</tr>
<tr>
<td>MATURITY STAGE</td>
<td>FISH TL (mm)</td>
<td>WT (gm)</td>
</tr>
<tr>
<td>----------------</td>
<td>-------------</td>
<td>---------</td>
</tr>
<tr>
<td>Immature</td>
<td>Up to 38</td>
<td>0.38-0.73</td>
</tr>
<tr>
<td>Ripening</td>
<td>42-55</td>
<td>0.65-2.18</td>
</tr>
<tr>
<td>Ripe</td>
<td>50-75</td>
<td>1.75-2.45</td>
</tr>
<tr>
<td>Spent</td>
<td>53-60</td>
<td>2.07-2.25</td>
</tr>
</tbody>
</table>
Figure- 25  *Aplocheilus lineatus* : Frequency distribution of spermatogenic stages in different length groups
Table 27. *Aplocheilus lineatus*: Histomorphological features of spermatogenic cycle.

<table>
<thead>
<tr>
<th>CYTOLOGICAL STAGE</th>
<th>SPERMATOGENIC STAGE</th>
<th>CYTOMORPHOLOGY</th>
<th>GERMINAL CELL DIFFERENTIATION</th>
<th>INTERSTITIAL CELLS</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Spermatogonia</td>
<td>Small, thick-walled</td>
<td>Large number of spermatogonia along periphery. Growing spermatocytes found.</td>
<td>A few inert cells</td>
</tr>
<tr>
<td>II</td>
<td>Spermatocyte</td>
<td>Slightly increased in diameter, thin-walled; tubules were solid</td>
<td>Spermatocytes in clusters, with few spermatids.</td>
<td>Proliferation of cells</td>
</tr>
<tr>
<td>III</td>
<td>Spermatid</td>
<td>Greatly enlarged wall became very thin.</td>
<td>Germ cells in various stages of active spermatogenesis; spermatids in plenty, with a few spermatozoa; least number of spermatogonia and spermatocytes.</td>
<td>Interstitial cells showed a moderate activity</td>
</tr>
<tr>
<td>IV</td>
<td>Spermatozoan</td>
<td>Highly distended</td>
<td>Filled with spermatozoa and seminal fluid; sperm maturation stages; a few spermatids found.</td>
<td>Slightly more prominent</td>
</tr>
<tr>
<td>V</td>
<td>Post-spermiation</td>
<td>Thick-walled and empty</td>
<td>Sperm resorption; spermatogonia abundant; spermatocytes absent.</td>
<td>Highly developed and very active.</td>
</tr>
</tbody>
</table>
Plate 10. Testicular development of the killifish Aplocheilus lineatus

a. Immature testis showing germinal epithelium (ge), spermatogonia (sg) and spermatocytes (sc). X 100 HE.

c. Maturing testis Note: germinal epithelium (ge), spermatogonia (sg), spermatocytes (sc) and testicular tubule (tt) X 200 HE.

e. Maturing testis showing clusters of spermatogonia (sg) and spermatocytes (sc) X 1000 HE.

g. Mature testis showing abundance of sperms (sp) and lumen (l) X 1000 HE.

b. Mature testis showing germinal epithelium (ge), spermatogonia (sg) and testicular tubule (tt) X 100 HE.

d. Maturing testis Note: germinal epithelium (ge), spermatogonia (sg), spermatocytes (sc) and sperms (sp) X 400 HE.

f. Maturing testis showing abundance of spermatocytes (sc) and spermatids (st) X 1000 HE.

h. Mature testis showing abundance of spermatogonia (sg), spermatids (st), sperms (sp) and spermatocytes (sc) X 1000 HE.
Plate 11. Spermatogenesis of *Aplocheilus lineatus*

- **a.** Immature testis showing clusters of spermatogonia (sg), spermatocytes (sc) and myoid cells (my) SL: 25mm, X1000,HE.
- **b.** Immature testis showing cluster of spermatogonia (sg), spermatocytes (sc) and myoid cells (my) SL: 30mm, X1000,HE.
- **c.** Maturing testis showing maturation of spermatids (st) and spermatozoa (sz). SL: 37mm, X1000,HE.
- **d.** Maturing testis showing maturation of spermatids (st) and spermatozoa (sz). SL: 35mm, X 1000,HE.
- **e.** Mature testis showing seminiferous tubules filled with abundant spermatozoa (sz). SL: 55mm, X 200,HE.
- **f.** Partially spent testis showing few empty spaces in lobular lumen (l) with few spermatozoa. SL: 65mm, X 1000,HE.
- **g.** Spent testis showing low counts of spermatozoa. SL: 70mm, X 1000,HE.
- **h.** Spent testis showing many empty spaces in lobular lumen (l) with very few spermatozoa SL: 68mm, X 400,HE.
1. **Spermatogonia:** The testicular tubules were small, thick-walled. A large number of primary spermatogonia were seen along the periphery (Plate 10a, b, c). A few growing spermatocytes and a few inert interstitial cells were observed.

2. **Spermatocyte:** The diameter of the seminiferous tubules increased slightly and their walls became thin and solid (Plate 10a, c, f). Spermatocytes were present in clusters. A few spermatids were also seen.

3. **Spermatid:** The seminiferous tubules were enlarged and their walls became thin. The tubules were solid and filled with cysts of germ cells at various stages of spermatogenesis (Plate 10f, h). Spermatids were found in plenty, with a few spermatozoa in the middle region.

4. **Spermatozoa:** The spermiation-stage testes were characterized by well formed sperm ducts filled with spermatozoa and seminal fluid. Stages of matured sperms, spermatocytes and spermatids formed a lining to the ducts (Plate 11c, d, e, f). The interstitial cells were slightly prominent.

5. **Post spermiation:** Spent testes of *A. lineatus* exhibited evidence of sperm resorption. The presence of a number of empty tubules was an indication that spermiation had already occurred (Plate 11f, g, h). The interstitial cells were maximally developed and were very active. A few spermatids or spermatozoa were also found.

### 3.3. Discussion

#### 3.3.1. Ovarian cycle

The pattern of gonadal development of *A. lineatus* was similar to that of many teleosts (Sterba, 1983; Sakurai *et al.*, 1993; Nakatsura and Kramer, 1982; Monaco *et al.*, 1978; Chellappa *et al.*, 2003). The assessment of the breeding period in *A. lineatus* was based on the observation of gonadal maturation as in many fishes like *Betta splendens* (Sterba, 1983; Sakurai *et al.*, 1993), *Hyphessobrycon pulchripinnis* (Nakatsura and Kramer, 1982), *Adinia xenica* (Koenig and Livingston, 1976), *Aphanius iberus* (Delgado *et al.*, 1988), *Fundulus diaphanus* (Chippett, 2003) and *Fundulus luciae* (Kneib, 1978).
The histological observation of the monthly samples of the ovary of *A. lineatus* revealed that it had an asynchronous ovary showing various developmental stages of oocytes at all the seasons. This type of ovary was recorded in *Adinia xenica* (Able, 1984), *Cynopoecilus melanotaenia* (Arenzon et al., 2001; 2002), *Fundulus grandis* (Greeley and Mac Gregor, 1983), *Fundulus heteroclitus* (Able and Hagan, 2003) and *Lucania goodie* (Fuller, 2001; 2002). Heins and Baker (1988) reported that oocytes of all sizes were present in *F. heteroclitus* and eggs ovulated continuously in an asynchronous ovary during the spawning season (Selman and Wallace, 1986). Monaco *et al.* (1978) reported that the presence of oocytes at various stages of development occurred simultaneously in *Poecilia mexicana*.

It was assumed that in fish gonads, the maturation of the germ cells coincided with increase in gonad weight (Stoumboudi *et al.*, 1993). However, the process of gonad ripening was not identical in males and females. The oocytes began to grow during the prophase of the first meiotic division and continued to accumulate metabolites until ovulation, thereby increasing the weight of the female gonad (Stoumboudi *et al.*, 1993). The development of gonads in *A. lineatus* was correlated with somatic growth. Maximum gonadosomatic index value was obtained in the gravid ovary. So the GSI values of *A. lineatus* corresponded closely with gonadal development as in *F. heteroclitus* (Hsiao *et al.*, 1994; Wallace and Selman, 1981), *F. grandis* (Hsiao and Meier, 1988) and *Aphanius iberus* (Delgado *et al.*, 1988).

Spawning in *A. lineatus* was represented by a decrease in ovarian weight as in *Fundulus heteroclitus* (Hsiao *et al.*, 1994), *Aphanius iberus* (Delgado *et al.*, 1988) and *Fundulus diaphanus* (Chipett, 2003).
3.3.2. Size frequency distribution of oocytes

The monthly record of the oocytes in *A. lineatus* showed the presence of numerous mature pre-ovulatory follicles in almost all the months especially in ripe ovaries. The same trend was observed in bayou killifish *Fundulus pulverous* and diamond killifish *Adinia xenica* (Greeley, 1984). Monaco *et al.* (1978) reported that female *Poecilia mexicana* were ovoviviparous and exhibited super-foetation, as well as asynchronous development of oocytes. Greeley *et al.* (1988) reported 6 oocyte developmental stages in *Fundulus grandis* based on microscopic observation. Mature oocytes were present in the gonad of *A. lineatus* almost throughout the year, indicating a multiple spawning strategy. The mature oocyte was independent of fish length of females in *A. lineatus*. The same trend has been reported by Kneib (1978) in *Fundulus heterooclitos* and in *Adinia xenica* (Greeley *et al.*, 1988).

It was observed that the size of oogonia to hydrated oocyte in *A. lineatus* ranged from 85 - 925µm. Greeley and MacGregor (1983) reported that the average diameter of ovulated eggs was 2.0mm in *Fundulus grandis*. The regressing ovaries of *A. lineatus* were identified by the absence of hydrated oocytes and occurrence of numerous atretic oocytes. Greeley *et al.* (1988) reported that in *Fundulus grandis* regressing ovaries were characterized by the absence of healthy hydrated oocytes and the presence of many atretic oocytes. In *Aphanius iberus* three kinds of eggs such as the transparent recruitment eggs (< 0.4mm), white opaque eggs (0.5 - 0.75mm) and yolky eggs (0.75 - 1.75mm) were reported by Delgado *et al.* (1988).

3.3.3. Follicular atresia

It was observed that in *A. lineatus* the occurrence of atretic oocytes were more especially at the end of spawning. It may compensate or regain the nutritional condition
of the ovary that was lost during spawning. Koster et al. (2003) described follicular atresia as a commonly observed phenomenon in fish which was linked with the nutritional condition of the females. Follicular atresia occurred in fishes with unsatisfied metabolic requirements (Hunter and Goldberg, 1980; Melo, 1994). Kjesbu et al. (1991) reported that reduction in potential fecundity in Atlantic cod was due to follicular atresia in the pre-spawning ovary. Monaco et al. (1978) reported the occurrence of low frequency of follicular atresia in *Poecilia mexicana*. No significant differences in the levels of oocyte atresia were detected in comparison of *P. mexicana* and *P. formosa*. Greeley et al. (1988) reported that seasonal changes in *Fundulus grandis* were similar to *F. heteroclitus* (Mathews, 1938) and the seasonal cessation of spawning was followed by the atresia of vitellogenic or mature oocytes. Since *A. lineatus* was a multiple spawner, the occurrence of follicular atresia was almost the same in all the months. It was also observed that the gut was almost empty during the peak spawning period in *A. lineatus*. The same trend was observed by Wallace and Selman (1978) in *F. heteroclitus* and suggested that there was mobilization of nutrients from yolk-containing oocytes for the physiological needs of the starving female.

Macchi et al. (2003) reported that the number of hydrated oocytes decreased at the end of the breeding season, coinciding with an increase of follicular atresia in *Micropogonias furnieri*. Macchi et al. (2003) also observed high levels of follicular atresia in regressing ovaries and established the cessation of spawning in other species (Hunter and Macewicz, 1985; Hunter et al., 1986; Dickerson et al., 1992; Barbieri et al., 1994).
3.3.4. Testicular cycle

Testicular histology of teleosts had been described by Nayyar and Sundararaj (1970); Sehgal (1971); Grover (1971); Bisht (1974); Shrestha and Khanna (1978); Swarup and Srivastava (1979); Raina and Bali (1982); Billard (1983, 1987); Joshi and Joshi (1989), and Srivastava and Singh (1992). The entire testis was histologically identical to Coesius plumbeus (Ahsan, 1966), Clarias batrachus (Lehri, 1967), Gerra gotyla (Shrestha and Khanna, 1978) and F. heteroclitus (Hsiao et al., 1994).

The development of the testes in the present study was similar to that of Nothobranchius furzeri (Haas, 1976) and Aphanius iberus (Delgado et al., 1988). Among teleosts, the spermatogenic activity commenced at various months of the year (Dixit and Agarwal, 1974; Kaul and Rishi, 1986; Joshi and Joshi, 1989; Srivastava and Singh, 1992). In A. lineatus, the first meiotic division was actively initiated at the onset of breeding. This was confirmed by the presence of plenty of spermatocytes. It also exhibited many peaks of testicular maturity in a year as in F. heteroclitus (Wallace and Selman, 1981) and Aphanius iberus (Delgado et al., 1988).

A. lineatus also exhibited continuous spermatogenic cycle and passed through the successive stages of growth, maturation, activation, depletion and rest during the annual reproductive cycle. The spermatogenic activity started in late August, progressed through September to full maturity into spermatozoa at the end of October and April. Spermiation took place in October and late November and thereafter spermatogenesis slowed down considerably and almost ceased by the middle of January. Again spermatogenesis started in March and ended in June. The testes remained quiescent and passed through a period of rest from January to March and June to September. In general, the testes of fishes showed an intensive phase of spermatogenic activity followed by a period of
spermatogenic rest. However, in some freshwater fishes, spermatogenesis began as soon as the spermiation was terminated and then continued throughout the year (Sanwal and Khanna, 1972). Most of the Indian freshwater teleosts attained maturity and bred during the monsoon season (Hsiao and Meier, 1988; Blanchard, 1996; Espana et al., 1998; Delgado et al., 1988, Chippett, 2003). *A. lineatus* also spawned during the monsoon period.

Craig-Bennett (1931) observed seasonal proliferation of androgen-producing interstitial cells of the testes prior to spawning season in *Gasterosteus aculeatus*. Wiebe (1969) also observed seasonal changes in the cytomorphology of interstitial cells in relation to the testicular cycle in the perch, *Cymatogaster aggregata*. In *A. lineatus*, the interstitial cells exhibited cytological changes in correlation with the testicular cycle. The interstitial cells became active during the periods only when meiosis and spermiogenesis occurred in the seminiferous tubules. As the spermiation occurred, the activity of the cells slowed down substantially and remained passive till the next spermatogenic cycle. It thus appeared that the hypertrophy of the interstitial cells was related to their steroidogenic activity, which caused the initiation and completion of the spermatogenic cycle. However, further experimental studies relating hormonal assay are desirable to substantially prove this phenomenon.

In the testes, there was an initial increase in weight due to an intense mitotic activity of the spermatogonia. During the spermatogenic process, this augmentation in weight continued until spermiogenesis. At this time, a part of the germ cell cytoplasm, the so-called ‘residual body’ was eliminated from the cell and it became phagocytosed by Sertoli cells (Grier et al., 1980; Billard, 1983; Selman and Wallace, 1986; Hamaguchi, 1987). As a result, after an initial increase, the weight of the male gonad became reduced.
Additional processes occurred in the testes, such as hydration, expansion of the interstitial spaces and proliferation of cells, which contributed to the changes in their weight (Stoumboudi et al., 1993).

3.4. Summary

Thus investigation on the maturity and spawning of *A. lineatus* were carried out by examining the gonad development, sperm and oocyte maturation, frequency distribution of maturity stages and oocytes. It was concluded that *A. lineatus* had an asynchronous ovary and spawned throughout the year. Hence *A. lineatus* was considered a multiple spawner. The occurrence of hydrated oocytes on every alternate month indicated that there was asynchronous development of oocytes in *A. lineatus*. The testicular cycle showed that the maturation of males coincided with the maturation of females to enable successful fertilization and maintenance of the progeny.